TIRC Grant #181R1

Benjamin A. Rubin, Ph.D.
Baylor University

CONFIDENTIAL

Report No. 2

November, 1959

An Evaluation of the Phenomenon of Tumor Growth Enhancement as an Assay for Carcinogens Among the Polycyclic Hydrocarbons and Related Compounds

The major part of our efforts during the past year in this program were related to the study of the mechanism and extent of carcinogen effect on transplantation; and on the quantitation of techniques for administration of chemicals and the measurement of growth effects.

Since we are unable, for reasons of time and money, to raise enough of our own DBA/2 and C3H mice, we have resorted to buying these strains from the Jackson Memorial Laboratory. This created a problem in re-standardizing the growth of our tumors and quantitating the effects of the carcinogens. Several of our old lines of the 6C3HED lymphosarcoma carried in CBA mice grow poorly (subcutaneously) in both C3H/JAX and carcinogen treated DBA/2 (Jackson) mice. There is a good correlation between the extent of growth in these two host types.

In the course of other experiments we isolated several new tumor lines from painted DBA/2 mice in which regressed tumors spontaneously reappeared. These "selected" tumors were then transplanted into a variety of other mice, including normal and treated DBA/2, C3H, and hybrids between these strains, and studied both as subcutaneous and as ascites tumors. While these studies are still in progress, we have already developed one tumor line that grows very well in carcinogen treated DBA/2 but hardly at all in normal mice of that strain. The growth in C3H/JAX is not quite as good as in the treated DBA/2 mice. Further work on the standardization of this tumor is in progress.

It may be pointed out, however, that the effect of the carcinogens on the growth of this tumor is very clear and very great. This was demonstrated in experiments where we measured the dose response at different levels of carcinogen treatment. We were able to show a large effect with a total skin application of 1×10^{-6} moles of material, equivalent to 25µg of methylcholanthrene. This was the smallest amount used in the test, but was far above the level of detectability. (see attached photograph)

This finding may be compared with our earlier result (in the USPHS Report submitted last year) in which the same amount of methylcholanthrene was barely at the level of detectability in the previous system of testing. Another innovation, besides the use of a new tumor line, is instead of presenting the carcinogen in a single dose (titration by varying the number of applications of the same solution), it is now given in 10 divided doses over a period of three weeks. In this system the number of applications remains

constant, but the dilution of the test solutions is changed. The effect and significance of this technique will be discussed further in another context.

Another problem related to quantitation of the carcinogens concerns the question of chemical integrity. In discussions with Dr. Ralph Becker, he pointed out that some of the common polycyclic hydrocarbons were extremely labile. The question arises as to whether it is the pure hydrocarbon or its oxidation products that are carcinogenic. Becker feels that many substances of this type used in biological experiments are really only degradation products.

This possibility was tested here with 7,12 (9:10) dimethylbenzanthracene, one of the most easily oxidized compounds of our study. Solutions were prepared in spectroscopic grade benzene and then divided. Half was deoxygenated (with pure nitrogen) and kept in dark bottles at 5°C in the presence of polished iron wires. Samples of this were rapidly withdrawn for use and the residues never returned. The control (the other half of the same solutions) were kept in the usual dark bottles in the cold but without the other precautions. In tests lasting three weeks, the carefully handled materials had approximately twice the tumor growth enhancing effects of the control solutions.

It is therefore quite clear that our previous type of handling which is more careful than most biological testing methods described in the literature, is still quite inadequate for good quantitative comparison. The differences in biological effect can be greatly complicated (and perhaps obliterated) by the differences in chemical reactivity. It is also clear that in the case of this compound, at least, it is the original material and not the oxidation products that has the biological effect.

In the course of studies on the mechanism of action of polycyclic hydrocarbons it became evident that some substances could potentiate the ability of these compounds to enhance tumor growth. If this could be refined to a consistent effect, it might not only help elucidate the mechanism of carcinogenesis but would make the quantitative determinations more sensitive. This is especially valuable where the hydrocarbon supply is very small and cannot easily be replaced.

This phenomen, of potentiating the carcinogen effect, was demonstrated in experiments using poorly growing tumors in DBA/2 mice that had been painted with different levels of methylcholanthrene. In this design, one could detect either a depression or an increase in the effect of the carcinogen.

The most striking effect was produced by Kinetin, and a smaller effect was also seen with Zymosan and Vitamin B12. All of these agents are thought to enhance immunological response. These observations were contrary to the concept held by some people that carcinogens depress the immune response. If this were true, antibody stimulating mechanisms should reverse carcinogen effect. In view of our results we must consider the possibility, suggested by H. N. Green and others, that carcinogens may

actually stimulate an antibody response that can interfere with the animals normal immunity -- possibly by a mechanism similar to the "enhancement" described by Kaliss and others.

In this context the action of corticoids is difficult to interpret. While cortisone alone, given before the tumor has a growth enhancing effect, it has the opposite effect if it is presented after the tumor is already implanted. When given at the same time as the carcinogen, there was a marked synergistic effect on the growth of a tumor that failed to respond to the enhancing effect of the same dose of either agent alone. However, the characteristics of the tumor growth were different from that seen after carcinogen treatment alone (USPHS Report). The tumor tends to persist rather than grow and the animals show no development of immunity. Subsequent tumor implants can grow as well or better than the original tumors.

Another type of observation also supports the antibody stimulating effect concept. Different strains of mice were treated with carcinogens for various periods and then sacrificed. Their RE tissues, blood, marrow, kidneys, etc. were examined histologically. The most consistent finding was the early stimulatory effects on the lymph nodes and RE tissues in general. This was manifested by hyperplastic enlargement of the nodes and evidence of rapid proliferation of the lymphocytic elements.

It can now be pointed out again that the same amount of a carcinogen when given in divided doses over three weeks has more tumor enhancing effect than a single dose. This too might be interpreted as fitting the pattern of an immunological effect.

The continuation of our studies using the transplantation of skin as a criterion for the carcinogen effect has several advantages over the tumor studies. It is not possible, for instance, to test the effect of continued carcinogen treatment because of the interference with tumor growth. Similarly the effect of anti-metabolites which might interfere with antibody production also have anti-tumor effects. Progressive tumor growth also creates a maximum effect beyond which no further observation can be made. As we have previously reported, we can greatly improve the transplantability of homologous skin by carcinogen treatment. This effect was further improved by treating animals with Amethopterin after the foreign skin was already implanted. No improvement of effect was obtained by continuing treatment with the carcinogen after transplantation. Further experiments are in progress (based upon extension of the design, using antibody stimulating methods during pretreatment and suppressing agents after the skin is implanted) to determine what combination of treatments will extend the period of successful transplantation.

Another line of experimentation, (we reported this at the last Cancer Meeting) deals with the effects of carcinogen treatment on animals in parabiosis. Here too the findings support the idea that the carcinogen effect is producing a new characteristic in the animals, rather than just suppressing antibody response. These experiments were rather complex in concept, execution, and interpretation but the general conclusion could be summarized thus:

1) Antibody goes across the parabiotic barrier. This is indicated by the immunity of an animal that has itself had no previous tumor.

2) A normal animal in parabiosis with a carcinogen treated one does not transfer its natural protective mechanisms to the susceptible (carcinogen painted) partner.

3) In the same combination, a normal animal becomes more susceptible to tumor growth, when in parabiosis with a treated mouse.

4) Tumor growth can be further stimulated in the normal animal by giving "enhancing" substances (in form of secondary tumor transplants) to the treated partners.

Summary

The work of the past year dealt with the standardization of tumor growth and response to carcinogens. The sensitivity of the tumor enhancing effect was increased both by use of new tumor-mice combinations as well as by alterations in technique. These changes arise from experiments pointing to a new interpretation of the effect in terms of increased immune (enhancing) response caused by the carcinogens. A new system of handling of carcinogens to avoid chemical change (oxidation) was developed, based on the finding that the usual precautions were inadequate and lead to a loss of biological effectiveness.

Plans for Future Studies

We are in the process of testing a variety of other tumor types and lines for their growth in carcinogen treated animals. Within the next three months or so we hope to have several stabilized systems with varying levels of sensitivity.

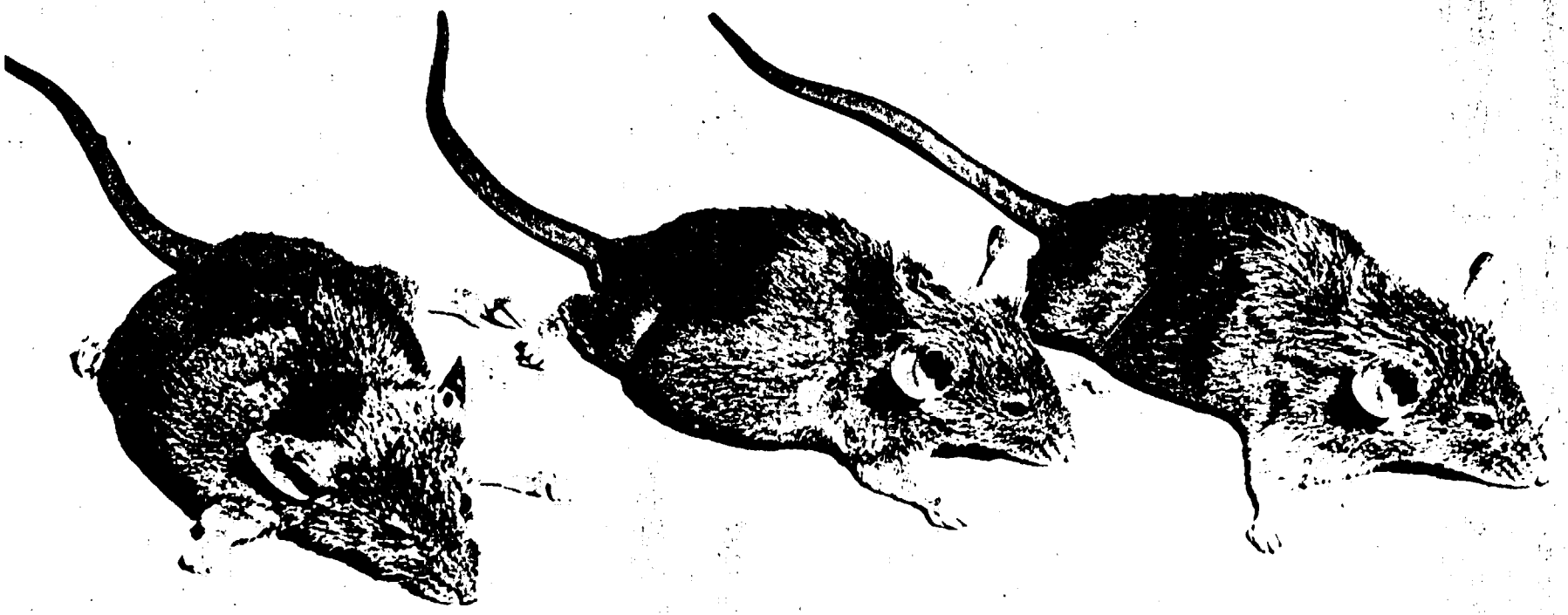
We also have in progress a large group of experiments using different substances that are shown to affect the carcinogen response. In one direction these findings may be used to further increase the quantitative response (as well as help elucidate the mechanisms involved). On the other hand, the possibility of interfering with carcinogens by a pharmacological method might have very great practical applications.

We are also working out detailed schedules of administration of the carcinogens to produce a predicable dose-response relation with agents of known effect. This is necessary before any quantitative comparison between different substances is really valid.

We hope that by mid-year most of these problems will have been resolved well enough to permit a large scale comparison of some of our more valuable analogous series of hydrocarbons.

We plan to work with Dr. Becker of the University of Houston in an attempt to correlate biological and chemical and properties; and also with Dr. Boutwell of University of Wisconsin to provide a comparison with a sensitive version of a classical test of carcinogenesis.

Experiments also continue on the effect of carcinogens on the transplantation of normal tissues. This is proving to be extremely promising from a theoretical as well as a practical viewpoint. Currently experiments are also underway on the homogologous transplantation of bone marrow, as a further extension of the utility of these effects of the polycyclic hydrocarbons.



TUMOR OF C3H ORIGIN GROWING IN DBA/2 MICE

Partly shaved ♂ DBA/2 mice 14 days after subsutaneous implantation of the 6C3HED Lymphosarcoma (on the right side). The mouse on the left had previously been treated with $1x10^{-5}$ moles of 3-methylcholanthrene, the center mouse had $1x10^{-6}$ moles by the same schedule. The animal on the right is an untreated control showing detectable tumor growth. For immobilization the mice had received 15 mg/kg of Thorazine.

100354030